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## Short communication

## Prolonged subordination stress increases Calbindin-D28k immunoreactivity in the rat hippocampal CA1 area

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**Abstract**

Previously we observed that corticosteroids alter Calbindin-D28k immunoreactivity in the rat hippocampus. In the present study we investigated whether prolonged subordination stress, presumably producing elevated plasma corticosterone levels (1) altered the immunocytochemical distribution of the  $\text{Ca}^{2+}$ -binding proteins Calbindin-D28k (CBir) and Parvalbumin (PVir) in the rat hippocampus, and (2) induced ongoing neurodegenerative changes using a silver impregnation method. Eight days of subordination stress reduced body weight, increased adrenal weight corrected for body weight and reduced thymus weight, indicating its effectiveness to produce a stressful situation. Stress increased CBir selectively in the CA1 pyramidal cell layer whereas PVir was not altered. Silver-impregnation revealed no ongoing neurodegenerative changes in any of the hippocampal subfields.

**Keywords:** Social stress; Hippocampus; Calcium; Calcium-binding protein; Calbindin-D28k; Parvalbumin

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Adult rats exposed to supraphysiological levels of corticosteroids display atrophy of the apical dendrites of their hippocampal CA3 pyramidal neurons [36]. Longer periods of exposure to corticosteroids may cause loss of hippocampal neurons [25] (but see also [2]) and shorter exposure to corticosterone increases the vulnerability of hippocampal neurons [9,26,28,29]. Similarly, three weeks of restraint stress causes shrinkage of the apical tree of hippocampal CA3 pyramidal neurons in rats [18,31,34,35], exposure to longer periods of stress may cause hippocampal neuron loss [21,33] and shorter periods of stress appear to endanger hippocampal neurons [30].

It has been hypothesized that glucocorticoids and stress increase hippocampal neuronal vulnerability by elevating extracellular glutamate levels which may, via ionotropic glutamate receptors, result in elevated (deleterious) intracellular  $\text{Ca}^{2+}$  levels ( $[\text{Ca}^{2+}]_i$ ) [8,9,23,29,30]. Additionally electrophysiological evidence indicates that corticosteroids increase voltage-gated  $\text{Ca}^{2+}$  currents [12].

However, there is also evidence that high levels of

corticosteroids increase the capacity to maintain neuronal  $\text{Ca}^{2+}$  homeostasis. High levels of corticosteroids increase the immunoreactivity [14,15], protein levels, and mRNA levels [11] of Calbindin-D28k (CB) which can act as intraneuronal  $\text{Ca}^{2+}$  buffer [16,19,20,27]. In the present study we firstly investigated whether elevation of plasma steroid levels by physiological rather than pharmaceutical treatment alters the immunoreactivity of the calcium binding protein CB and Parvalbumin (PV). Therefore we studied the effect of prolonged subordination stress induced by exposure of experimental animals with a dominant male rat on the immunocytochemical distribution of CB and PV. Secondly we studied whether this stressor induced degenerative changes in the hippocampus using a sensitive marker for ongoing neurodegenerative changes [13,14,22,32].

The animal experiments were approved by the Committee on Animal Bio-Ethics of the University of Groningen. Twenty two male Tryon Maze Dull (TMD S3) rats of 4 months of age (originally derived from Cpb, TNO Zeist and locally bred at the department of Animal Physiology, University of Groningen, the Netherlands) were kept in groups (6–7 animals per cage) on a 12 h light–dark schedule (7:00–19:00 dark). Food and water were available ad libitum. At the start of the experiment the animals

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were divided into three groups. Seven rats were confronted with a selected dominant male TMD S3 rat (Stress group). The confrontation took place in a wooden cage ( $85 \times 60 \times 50$  cm) which was permanently occupied by the dominant rat. The social interaction resulted in display of submissive behaviour of the experimental animal. After submission, the experimental rat was put into a nestbox and replaced in the cage of the dominant rat for eight days. The experimental animal had to leave its nestbox to get food and water. Additionally, seven animals were individually caged for eight days in a similar wooden cage ( $85 \times 60 \times 50$  cm) without confrontation with a male dominant rat (control), and eight animals were kept in a group (naive). All experimental groups were kept in the same room.

Eight days after the start of the experiment the animals were perfused. Prior to transcardial perfusion rats were anaesthetized with pentobarbital (50 mg/kg i.p.) and perfused with 0.9% saline for 1 min, followed by 4% paraformaldehyde (PF) in 0.1 M phosphate buffer (PB, pH 7.4) for 10 min. Brains were removed from the skull and postfixed in 4% PF for 24 h, before being stored in 50 mM Tris buffered saline (pH 7.4) containing 0.1% sodium-azide. The adrenals and thymuses were removed and weighed. Brains were cryoprotected by overnight storage in 30% sucrose in 0.1 M phosphate buffer (PB, pH 7.4) and cut into 30  $\mu$ m thick coronal sections at  $-15^\circ\text{C}$  using a cryostat microtome. Sections were collected in 50 mM Tris-NaCl, pH 7.4, containing 0.1% sodium-azide.

Immunocytochemical staining for CB and PV was performed on free floating sections as described below, all steps being identical and run simultaneously. Tissue sections were rinsed in PBS containing 0.2% Triton X-100 (TX-PBS), rinsed in PBS and subsequently preincubated for 30 min in 0.3%  $\text{H}_2\text{O}_2$  in PBS. Sections were then rinsed in PBS followed by incubation in 0.1% bovine serum albumin (BSA) in TX-PBS to suppress aspecific antibody binding to the tissue. Then, brain sections were incubated overnight at  $4^\circ\text{C}$  with the primary monoclonal antibodies (from Sigma) mouse IgG anti-Calbindin-D28k (1:400 dilution in PBS) or mouse IgG anti-Parvalbumin (1:2000 dilution in PBS). These antibodies have extensively been described and recognize the calcium-bound formation of CB and PV [3,4]. After rinsing with TX-PBS and PBS, sections were incubated in biotinylated sheep-

anti-mouse IgG (Amersham, diluted 1:200 in TX-PBS–0.1% BSA) for 3 h at room temperature (RT) followed by rinsing with PBS, TX-PBS and PBS. Sections were then incubated for 2 h in streptavidin-HRP (Zymed, 1:200 in PBS) at RT, rinsed in TX-PBS, PBS and Tris buffer (Tris, 0.05 M, pH 7.4). Finally the tissue bound peroxidase was visualized by reaction with diaminobenzidine (30 mg/100 ml Tris) and staining was started by adding 30  $\mu$ l 1%  $\text{H}_2\text{O}_2$  to the sections, and stopped when sections showed sufficient staining by rinsing in Tris and PBS. After staining the sections were mounted on gelatin–chrom–alumin coated slides, air dried, dehydrated in graded ethanols and xylol and coverslipped with DEPEX (BDH). The intensity of immunolabelling for CB and PV in the hippocampus was quantified at the level I.A. 5.7 (according to Paxinos and Watson [24]) by measuring the optical density (OD) using an image analysis system (Zeiss, IBAS). For CBir, the OD of the CA1 pyramidal cell layer, the DG granular cell layer and molecular layer, as well as the OD of the mossy fiber pathway was calculated. For PVir we calculated the OD of the CA1 and CA3 pyramidal cell layer and the DG granular cell layer. As control for background staining of CB and PV we measured the stratum oriens of the CA3 area and the corpus callosum respectively which are devoid of immunoprecipitate. The OD of the area of interest was related to the background value by the formula  $[(\text{OD}(\text{area}) - \text{OD}(\text{background})) / \text{OD}(\text{background})] \times 100$ , thus eliminating the variability in background staining among sections [7]. Four sections per animal were quantified. Statistical analysis was performed using the Mann–Whitney  $U$  test. Significance level was  $P < 0.05$ .

The modified Gallyas silver impregnation procedure of Nadler and Evenson [22], which labels degenerating cell bodies and their processes, was employed as described previously [13,32]. Sections were rinsed  $3 \times 5$  min in distilled water, pretreated  $5 \times 5$  min in 4.5% NaOH and 0.6%  $\text{NH}_4\text{NO}_3$ , and impregnated for 10 min in 5.4% NaOH, 6.4%  $\text{NH}_4\text{NO}_3$  and 0.3%  $\text{AgNO}_3$ . Following this step sections were rinsed  $3 \times 5$  min in 0.5%  $\text{Na}_2\text{CO}_3$  and 0.012%  $\text{NH}_4\text{NO}_3$  in 30 ethanol. Next, the sections were developed in 0.0057% citric acid, 0.55% formalin, 9.6% ethanol, 2.7% NaOH and 0.006%  $\text{NH}_4\text{NO}_3$ . Sections were fixed for 4 min in 37.5%  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  and rinsed

Table 1  
Effect of eight days subordination stress on body weight, adrenal weight and thymus weight

Treatment	<i>n</i>	BW <sub>i</sub> (g)	BW <sub>a</sub> (g)	dBW(g)	Adrenal weight (mg)	Adrenal weight/100 g (mg)	Thymus weight (mg)	Thymus weight/100 g (mg)
Control	7	328.4 $\pm$ 2.2	349.4 $\pm$ 2.1	21.0 $\pm$ 2.6	22.95 $\pm$ 1.5	6.56 $\pm$ 0.41	261.3 $\pm$ 11.5	74.8 $\pm$ 3.2
Stress	7	340.0 $\pm$ 7.2	313.3 $\pm$ 8.0	–27.1 $\pm$ 2.4 <sup>ab</sup>	27.59 $\pm$ 2.4	8.80 $\pm$ 0.80 <sup>ab</sup>	156.8 $\pm$ 17.9 <sup>ab</sup>	50.0 $\pm$ 5.5 <sup>ab</sup>
Naive	8	335.1 $\pm$ 7.1	350.8 $\pm$ 8.2	15.6 $\pm$ 5.0	22.22 $\pm$ 1.16	6.33 $\pm$ 0.28	268.4 $\pm$ 25.8	77.5 $\pm$ 8.1

Animals suffering eight days subordinations stress ( $n = 7$ ) show a significant reduction in body weight, increased adrenal weight corrected for body weight, and reduced absolute and relative thymus weight when compared to both control ( $n = 7$ ) and naive ( $n = 8$ ) animals.

<sup>a,b</sup> Indicate  $P < 0.05$  when compared to control and naive animals respectively.

BW<sub>i</sub>: body weight measured immediately prior to the stressor; BW<sub>a</sub>: body weight measured immediately prior to perfusion; dBW: change in body weight.

3 × 5 min in distilled water, mounted and coverslipped. For details of the procedure see [32].

The prolonged subordination stress had profound effects on body weight (Table 1). During the eight days experimental period both control and naive animals gained body weight ( $21.0 \pm 2.6$  g, and  $15.6 \pm 5.0$  g respectively) whereas the stressed animals lost body weight when compared to their weight immediately prior to the stress period ( $-27.1 \pm 2.4$  g). Stress increased the adrenal weight when corrected for body weight, but did not increase absolute adrenal weight when compared to control and naive animals ( $P = 0.1792$  and  $0.0930$  respectively). However, both the absolute and relative thymus weight of the stressed animals were reduced when compared to the control and naive animals.

In control rats DG granular cells were intensely immunoreactive for CB, as were their dendrites in the stratum moleculare. Also the mossy fiber pathway was stained, whereas pyramidal cells in the inner half of the CA1 pyramidal cell layer showed moderate CBir, while their dendrites in the stratum radiatum were weakly labelled. A small number of interneurons was present mainly in the stratum radiatum of CA1–CA3. Eight days continuous subordination stress increased CBir exclusively in the CA1 pyramidal cell layer (Fig. 1).

PVir was selectively present in non-principal neurons in all hippocampal regions, which is in agreement with earlier descriptions [5]. In the CA1 field almost all PVir pericarya were situated in the pyramidal cell layer and stratum oriens

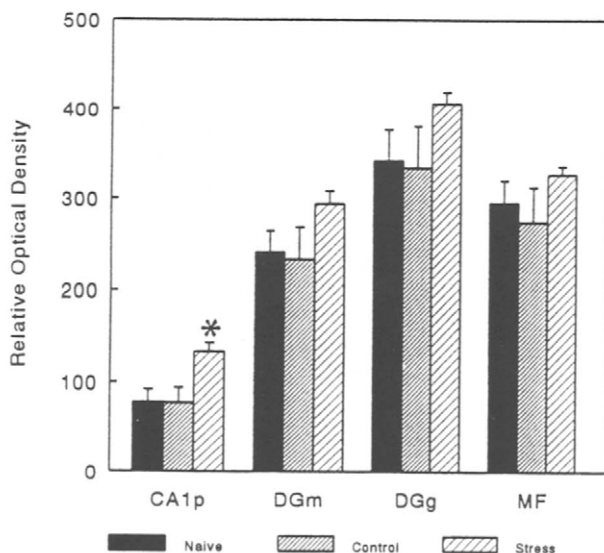


Fig. 1. Relative optical density of Calbindin-D28k in the rat hippocampal subfields of stressed, naive and control animals. Immunoreactivity of Calbindin-D28k was significantly increased in the CA1 pyramidal cell layer of stressed ( $n = 7$ ) animals when compared to both control ( $n = 7$ ) and naive ( $n = 8$ ) animals. \* indicates  $P < 0.05$ . CA1p = pyramidal cell layer in the hippocampal CA1 field; DGm = molecular layer of the dentate gyrus; DGg = granular cell layer of the dentate gyrus; MF = mossy fiber layer.

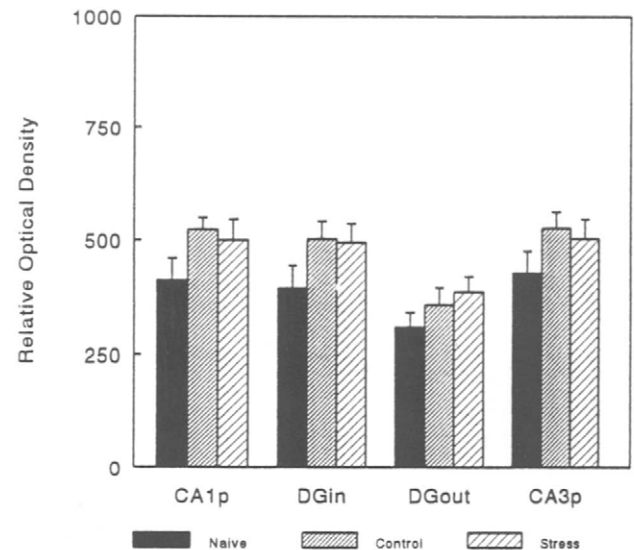


Fig. 2. Relative optical density of Parvalbumin in the rat hippocampal subfields of stressed ( $n = 7$ ), naive ( $n = 8$ ) and control ( $n = 7$ ) animals. No alterations were observed in any of the studied subfields between the groups. ( $P < 0.05$ ). CA1p = pyramidal cell layer in the hippocampal CA1 field; DGin = hidden blade of the dentate granular layer; DGout = outer blade of the dentate granular layer; CA3p = pyramidal cell layer in the hippocampal CA3 subfield.

with their varicose dendrites extending into the stratum radiatum and stratum oriens. The axons of PVir interneurons enmeshed the cell bodies of the immuno-negative CA1 pyramidal cells. In the dentate gyrus most of the PVir cells were situated in the sub- and supragranular layers and hilar region while their dendritic processes could be followed into the hilus and molecular layers. Moreover, the granular cells were embedded in a dense network of PVir axon terminals. The eight days continuous subordination stress did not alter PVir in any of the hippocampal subfields (Fig. 2).

Silver impregnation revealed no signs of somatic or dendritic degenerative changes in any of the hippocampal subfields of stressed animals (photographs not shown).

The present study shows that (1) prolonged subordination stress alters parameters that have traditionally been associated with prolonged stress, (2) stress increases Calbindin-D28k immunoreactivity in the rat hippocampal CA1 area, and (3) the silver-impregnation method revealed no degenerative changes in the rat hippocampus following eight days of subordination stress.

Our results demonstrate that eight days of subordination stress alters parameters that have been associated with prolonged stress [1,6] indicating that the procedure induced a stressful situation for the rats. Animals that were exposed to this stress-paradigm displayed increased Calbindin-D28k immunoreactivity exclusively in the hippocampal CA1 pyramidal cell layer whereas PV immunoreactivity in the hippocampus was not altered by the stress. Previously we

observed that prolonged exposure to high levels of corticosterone was associated with increased CB-immunoreactivity exclusively in the hippocampal CA1 pyramidal cell layer [14,15] which probably reflects increased protein levels of Calbindin-D28k [11]. Since the stress used at present transiently elevates plasma corticosterone levels (unpublished results) one might speculate that (1) corticosterone mediates the increased Calbindin-D28k immunoreactivity following prolonged stress, and (2) the increased immunoreactivity following stress reflects increased protein levels.

In vitro studies have indicated that cells expressing CB exhibit lower  $\text{Ca}^{2+}$ -entry through voltage-dependent  $\text{Ca}^{2+}$  channels and are better able to reduce  $[\text{Ca}^{2+}]_i$  transients evoked by voltage depolarizations [16,19,27]. Therefore if the stress-induced elevation in Calbindin-D28k immunoreactivity reflects increased protein levels, one might expect that the  $\text{Ca}^{2+}$  buffering capacity of these cells is increased. During stress, plasma corticosteroid levels rise and such elevated plasma corticosterone levels increase voltage-gated  $\text{Ca}^{2+}$  currents in vitro [12]. One might speculate that increased Calbindin-D28k levels serve as an adaptation to elevated  $\text{Ca}^{2+}$  currents e.g. when plasma corticosterone levels are high.

Three weeks of restraint stress induces shrinkage of the apical dendrites of CA3 pyramidal neurons [18,31,34,35] and Fuchs et al. ([10], unpublished observations) observed similar alterations in the hippocampus of the tree shrew. Since the atrophy is a reversible phenomenon ([17], unpublished observations, [18] unpublished observations) it is not likely that the dendritic atrophy reflects early signs of neuron loss. This is supported by findings of Luine et al. [17] who reported that spatial deficits following three weeks of stress, which were accompanied with the dendritic atrophy, was also reversible. Furthermore Sunanda Roa and Raju [31] reported that the stress-induced shrinkage of the apical tree of CA3 pyramidal neurons was accompanied with an increased number of dendritic spines of apical and basal dendrites. In line with this we did not observe degenerative changes in the hippocampus following eight days of subordination stress at present using a sensitive marker for degenerative changes [13,22,32]. One might suggest that the eight days of stress is too short to produce degenerative changes but we also did not observe degenerative changes in the hippocampus after three weeks of daily changing stressors (unpublished observations). Our results however do not exclude that the presently performed subordination stress induces cytoskeletal changes in the rat hippocampus.

We conclude that the prolonged subordination stress as performed in the present experiment (1) is successful in producing a stressful situation and provides a useful model for studying stress-related phenomena, (2) increases Calbindin-D28k immunoreactivity in the rat hippocampal CA1 pyramidal cells, and (3) does not induce ongoing neurodegenerative changes in the rat hippocampus.

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